Effects of cadmium and phenanthrene mixtures on aquatic fungi and microbially mediated leaf litter decomposition

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Abstract

Urbanization and industrial activities have contributed to widespread contamination by heavy-metals and polycyclic aromatic hydrocarbons, but the combined effects of these toxics on aquatic biota and processes are poorly understood. We examined the effects of cadmium (Cd) and phenanthrene (Phe) on the activity and diversity of fungi associated with decomposing leaf litter in streams. Leaves of *Alnus glutinosa* were immersed for 10 days in a unpolluted low-order stream in the NW Portugal to allow microbial colonization. Leaves were then exposed in microcosms for 14 days to realistic concentrations of Cd (0.06 - 4.5 mg L^{-1}) and phenanthrene (0.2 mg L^{-1}) either alone or in mixtures. A total of 19 aquatic hyphomycete species were found sporulating on leaves during the whole study. The dominant species was *Articulospora tetracladia*, followed by *Alatospora puchella*, *Clavatospora longibrachiata* and *Tetrachaetum elegans*. The exposure to Cd and phenanthrene decreased the contribution of *A. tetracladia* to the total conidium production while increased that of *A. pulchella.* Fungal diversity, assessed as DGGE or conidium morphology, was lowered by the exposure to Cd and/or phenanthrene. Moreover, increased Cd concentrations depressed leaf decomposition and fungal reproduction but did not inhibit fungal biomass production. The exposure to phenanthrene potentiated the negative effects of Cd on fungal diversity and activity, suggesting that more studies should be conducted in order to assess the effects of multiple stressors on aquatic biodiversity and stream ecosystem functioning, since it might correspond to more realistic approaches.

Introduction

In streams with low autotrophic production, plant-litter decomposition provides carbon and energy for the functioning of aquatic foodwebs (Allan and Castillo 2007). This carbon is first processed through microbial activity increasing the palatability of plant litter for aquatic invertebrates (Bärlocher 2005; Gessner et al. 2007). Among microorganisms, aquatic hyphomycetes play a key role in plant-litter decomposition in streams (Pascoal and Cássio 2004; Pascoal et al. 2005a) due to their ubiquity and ability to produce a variety of extracellular enzymes that degrade the complex plant cell-wall polysaccharides (Bärlocher 2005; Gessner et al. 2007).

Human activities from agriculture, mining and industry have contributed to the increase of contaminants entering streams that affect the activity and diversity of biotic communities, including detritus-based food webs (Niyogi et al. 2001; Pascoal et al. 2005a,b; Sridhar et al. 2001). Among pollutants, metals can have adverse effects on aquatic biota because of their toxicity and persistence in the environment (Rand et al. 1995). Even though aquatic hyphomycetes have been found in metal-polluted streams, the diversity and activity of this group of fungi is constrained by the presence of heavy metals (Bermingham et al. 1996; Niyogi et al. 2002; Pascoal et al. 2005b; Sridhar et al. 2005). The exposure of leaf-associated fungal communities to copper and/or zinc has been reported to alter the structure of aquatic hyphomycete communities and reduce fungal sporulation and leaf decomposition (Duarte et al. 2004, 2008a, 2009a). Moreover, metals, including Cd, inhibit the growth and reproduction of several species of aquatic hyphomycetes, but reproduction appears to be more sensitive than growth (Abel and Bärlocher 1984; Azevedo and Cássio 2010; Miersch et al. 1997).

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxins containing two or more benzene rings that are known to have carcinogenic and mutagenic properties (Chaudry

1994). These compounds are components of coal, crude oil and its derivatives and are ubiquitous in the environment as they are formed during forest fires, combustion of petroleum and incineration of waste. Several studies have shown that microbes have the ability to metabolize and biodegrade PAHs and can potentially be used for bioremediation of contaminated environments (D'Annibale et al. 2006; Johnsen et al. 2005; Juhasz and Naidu 2000). For instance, the bacterium *Pseudomonas putida* degraded about 70% of the initial amount of phenanthrene (Phe, 0.47 g L^{-1}) in 27 days (Cuny et al. 2004) and the aquatic hyphomycete *Heliscus lugdunensis* is reported to metabolize metabolites of complex PAHs, such as 1-naphthol (Augustin et al. 2006). PAHs do not dissolve easily in water but can bind strongly to particulate organic matter and accumulate in the sediments of rivers and lakes at high concentrations, afecting aquatic biota and humans through bioaccumulation (Gust and Fleeger 2006; Ribeiro et al. 2005; Scoggins et al. 2007).

In aquatic ecosystems, the simultaneous occurrence of PAHs and heavy metals is quite common (Gust and Fleeger 2006). While many studies have investigated the individual effects of these toxins on organisms, relatively few have considered their effects in mixtures, particularly at the community level. In this study, the effects of Cd and phenanthrene on the diversity and activity of aquatic fungal communities associated with decomposing leaves were evaluated. Alder leaves were incubated in a stream to allow microbial colonization and then were exposed to increasing concentrations of Cd (0.06 to 4.5 mg L^{-1}), in the absence or presence of a fixed concentration of phenanthrene (0.2 mg L^{-1}). The effects were evaluated on leaf mass loss and fungal biomass, sporulation and diversity. Although there is no data concerning the co-occurrence of metals and PAHs in Portuguese streams, in the Northwest of Portugal there are several industrial areas and pollution by both kind of toxins are likely to occur in freshwaters

(Gust and Fleeger 2006). Although lower concentrations of Cd have been found in the water column in streams of the Northwest Portugal (0.06 mg L^{-1}, Gonçalves 2001), than those used in the current study, in sediments we found concentrations between 0.02 and 144 Kg g^{-1} of volatile matter dry weight in the <63 µm sediments fractions (Soares et al. 1999). The variation of some physical and chemical characteristics (pH, salinity, redox potential and content of organic chelators) of the water may provoke the release of these metals back to the aqueous phase and higher concentrations of Cd are expected to occur in water column. We expect that Cd and phenanthrene will restrict fungal diversity and activity, and this will be more severe in mixtures with increasing Cd concentrations. However, we also expect that some species might be able to metabolize phenanthrene and thereby survive under the tested conditions.

Materials and Methods

Sampling site

The sampling site is located at Algeriz, a low-order stream in the Northwest of Portugal (41º35'N 8º22'W). The riparian vegetation is dominated by *Eucalyptus globulus* Labill., *Quercus robur* L., *Alnus glutinosa* (L.) Gaertn and *Rubus* sp.

Leaves of *A. glutinosa*, collected in October 2006, were air-dried and kept at room temperature until used. The leaves were leached in deionised water for 48 hours and cut into 12 mm diameter disks. Sets of 22 disks were placed into 42 fine-mesh bags (20 x 20 cm, 0.5 mm mesh size) to prevent invertebrate colonization. On 20th March 2007, leaf bags were immersed in the stream for 10 days to allow microbial colonization. An additional set of 3 bags were immersed for 15 minutes in the sampling site and the content used to estimate initial leaf dry mass and ergosterol concentration in the leaves.

At the time of leaf immersion, stream water had a temperature of 11.8 ºC, a pH of 6.9, a redox potential of -8 mV, a concentration of dissolved oxygen of 10.8 mg L^{-1} and a conductivity of 40 µS cm^{-1}, measured *in situ* with field probes (Multiline F/set 3 nº 400327, WTW). Stream water was collected in dark glass bottles, transported on ice to the laboratory and analyzed within 6 h for quantification of inorganic nutrients with a HACH DR/2000 photometer (Hach company, Loveland, CO, USA). Nutrient concentrations were: orthophosphate, 0.06 mg P-PO_4^{3-} L^{-1} (HACH kit, program 490); nitrate, 0.2 mg N-NO_3^- L^{-1} (HACH kit, program 355); nitrite, 0.005 mg N-NO_2^- L^{-1} (HACH kit, program 371); and ammonium <0.01 mg N-NH_3 L^{-1} (HACH kit, program 385). Additional stream water was collected for microcosm experiments.

Microcosms

In the laboratory, sets of 22 leaf disks were placed into 150 mL Erlenmeyer flasks with 70 mL of filtered (Macherey-Nagel MN-GF3, glass fiber filter membranes) and sterilized (120 ºC, 20 min) stream water. The microcosms were supplemented with cadmium chloride (Sigma) at final concentrations of 0.06, 0.6, 1.2, 3.6 and 4.5 mg L^{-1} Cd and phenanthrene (Fluka) at a final concentration of 0.2 mg L^{-1}, added alone or in mixtures (3 replicates). Phenanthrene was solubilized in ethanol at a final concentration of 0.3 %. Microcosms were incubated on a shaker (110 rpm, Certomat BS 3, Braun, Melsungen, Germany), at 15 ºC, in the dark. The microcosm solutions were changed every 4 days, and phenantrene concentration was checked daily by direct fluorescence spectrophotometry (LS 50 luminescence spectrometer, Perkin-Elmer, Foster city, CA, USA) using the fixed wavelength technique (Watson et al. 2004; Yang et al. 2003). Microcosms without added toxins and microcosms with ethanol, at the concentration used to solubilize phenanthrene, were used as controls (3 replicates).

After 14 days of exposure, all microcosms were sacrificed and leaf disks were freeze-dried and weighed (± 0.001 g) for determination of leaf dry mass remaining, and stored at -80 ºC for further assays. Sets of non-colonized leaf disks were used to estimate the initial leaf dry mass.

Fungal biomass

Concentration of ergosterol was measured to estimate fungal biomass associated with decomposing leaf disks. Sets of 8 disks, from each replicate microcosm, were heated (80 ºC, 30 min) in 0.8 % KOH-methanol for lipid extraction and the extract was purified by solid-phase extraction, according to Gessner (2005). Ergosterol was quantified by high-performance liquid chromatography (HPLC, Beckmann Gold System, Brea, CA, USA) using a LiChrospher RP18 column (Merck). The system was run isocratically with HPLC-grade methanol at 1.4 mL min^{-1} at 33 ºC. Peaks of ergosterol were detected at 282 nm and standard series of ergosterol (Sigma) in isopropanol were used to estimate ergosterol concentration in the samples.

Fungal diversity

After 10 and 14 days in microcosms, conidium suspensions were filtered (5 µm pore size; Millipore, Billerica, MA, USA) and the spores were stained with cotton blue in lactic acid. Conidia were counted and identified under a microscope (400x; Leica Biomed, Heerbrugg, Switzerland).

DNA was extracted from sets of 3 leaf disks (1 from each replicate) using an UltraClean Soil DNA kit (MoBio Solana Beach, CA, USA). The ITS2 region of fungal ribosomal DNA was amplified by PCR, with ITS3GC and ITS4 primers (White et al. 1990). The reaction mixture contained 4 µM of each primer, 1 µL (~50 ng) of DNA, 1.5 U of Taq

polymerase, 3 mM of $MgCl_2$, 2 mM of DNTPs and 1x of Taq buffer ($KCl:(NH_4)_2SO_4$) in a final volume of 50 µL. Fungal DNA amplification was carried out in an iCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) and PCR started with a denaturation of 2 min at 95 ºC, followed by 36 cycles of denaturation for 30 s at 95 ºC, primer annealing for 30 s at 55 ºC and extension for 1 min at 72 ºC. Final extension was at 72 ºC for 5 min (Nikolcheva and Bärlocher 2005; Duarte et al. 2008a).

PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE), using a DCode Universal Mutation Detection System (BioRad). Samples of ~750 ng DNA were loaded on 8 % polyacrilamide gel in 1x Tris-acetate-EDTA (TAE) with a denaturing gradient from 30 to 70 % (100 % denaturant corresponds to 40 % formamide and 7 M urea). The gel was run at 55 V, 56 ºC for 16 hours and was stained with 1 µg mL^{-1} of ethidium bromide (BioRad). The images were captured under UV light in a transiluminator Eagle Eye II (Stratagene, La Jolla, CA, USA).

Data analysis

Leaf mass loss, fungal biomass, and sporulation were expressed as percentage of control. To achieve normal distribution and homocedasticity, data were arcsine square root transformed (Zar 1996). Ethanol at the concentration used to solubilize phenanthrene did not significantly inhibit leaf mass loss, fungal biomass and sporulation (t-tests, $P > 0.05$; Zar 1996). The effects of Cd and phenanthrene on leaf mass loss and ergosterol concentration were tested by a two-way ANOVA (Zar 1996). A three-way ANOVA (Zar 1996) was used to test if Cd, phenanthrene and exposure time affected sporulation rates and the number of leaf-associated aquatic hyphomycete taxa. Dunnett's post-tests were done to test which treatments significantly differed from

control (Zar 1996). Statistical analyses were performed using Statistica 7.0 (Statsoft, Inc., Tulsa, OK, USA).

The DGGE gel was aligned and normalized using Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium). Each DGGE band was treated as an individual operational taxonomic unit (OTU) and the number of OTUs was used as a measure of fungal diversity. A correspondence analysis (CA) (Legendre and Legendre 1998) was used to ordinate treatments using fungal communities, based on sporulating species or OTUs (as relative intensity of each band in DGGE fingerprinting). The analyses were done using CANOCO 4.5 (Microcomputer Power, Ithaca, NY, USA).

Results

Effects of cadmium and phenanthrene on fungal activity

In the absence of Cd and phenanthrene, alder leaves lost approximately 56% of their initial dry mass (0.061±0.009 g) after a 10-day colonization in the stream and 14 days in microcosms. Fungal biomass was 339 µg ergosterol g^{-1} dry mass, and fungal sporulation rate on decomposing leaves was 2.94×10^5 and 0.31×10^5 conidia g^{-1} dry mass day^{-1} after 10 and 14 days, respectively.

Leaf mass loss was significantly affected by Cd, phenanthrene and the interaction between both toxins (2-way ANOVA, $P<0.01$ for all factors) (Fig. 1A). Significant inhibition effects were found following the exposure to Cd at high concentrations (≥ 3.6 mg L^{-1}; Dunnett's test, $P<0.05$) and to phenanthrene alone or in mixtures with Cd (Dunnett's test, $P<0.01$).

Fungal biomass on decomposing leaves was significantly affected by Cd and phenanthrene (2-way ANOVA, $P=0.001$ and $P=0.003$, respectively) (Fig. 1B). Significant inhibition effects were found by exposure to mixtures of phenanthrene and

Cd at the highest concentration (4.5 mg L^{-1}; Dunnett's test, $P<0.05$). It seems to exist a pattern that shows that there is a slight stimulation in biomass production with the lower cadmium concentrations, which is significant in one treatment (1.2 mg L-1).

Sporulation of aquatic hyphomycetes was significantly affected by the concentration of Cd and phenanthrene (3-way ANOVA, $P<0.001$, for both comparisons) (Fig. 1C) but not by the exposure time (3-way ANOVA, $P=0.1$). Moreover, interactions between phenanthrene and exposure time and between the two toxins were significant (3-way ANOVA, $P=0.008$ $P<0.001$, respectively; Fig. 1C). At both exposure times, sporulation rate was inhibited by Cd concentrations $\geq$ 3.6 mg L^{-1} (Dunnett's test, $P<0.05$) and by mixtures of Cd and phenanthrene at concentrations of Cd $\geq$ 0.06 mg L^{-1} (Dunnett's test, $P<0.05$).

Effects of cadmium and phenanthrene on the structure of fungal community

During the study, 19 species of aquatic hyphomycetes were found sporulating on alder leaves (Table 1). The exposure to increasing Cd concentrations led to a decrease in the number of fungal species, particularly in microcosms supplemented with the highest Cd concentration (4.5 mg L^{-1}), in which only 8 fungal species were observed (Table 1). *Articulospora tectracladia* was the dominant species in control microcosms, contributing to 40% to the total conidia production. The exposure to the toxins, especially in mixtures, led to a decrease in the contribution of *A. tetracladia* (Table 1). On the other hand, the exposure to Cd, alone or in mixtures with phenanthrene, increased the contribution of the co-dominant species *Alatospora pulchella*. *Lemmoniera aquatica* seemed to be tolerant of Cd, since its contribution increased with metal concentration, attaining 41.3% of the total conidia production at the highest Cd concentration (4.5 mg L^{-1}). Phenanthrene increased ca 5-times the contribution of

Tetrachaetum elegans to conidium production, in the absence or presence of the lowest Cd concentration. An unidentified species became co-dominant in mixtures with high Cd concentrations.

Denaturing gradient gel electrophoresis (DGGE) of DNA of fungal communities on decomposing leaves showed 18 OTUs in control microcosms after 14 days in (Table 1, Fig. 2). The exposure to increasing Cd concentrations led to a decrease in the number of OTUs, particularly in the presence of phenanthrene. Generally, higher fungal diversity was found from DGGE than from conidium identification.

The CA ordination of fungal communities based on conidium morphology and DGGE OTUs showed that the exposure to Cd and phenanthrene altered the structure of fungal community, with stronger effects for communities exposed to mixtures of both stressors (Fig. 3). The analysis of fungal communities based on conidium morphology showed that the first axis, explaining 58% of the total variance, distributed communities along the gradient of Cd concentration in the absence or presence of phenanthrene (Fig. 3A). The second CA axis explained 37% of the total variance and separated communities exposed to phenanthrene from all the others. The CA ordination or fungal communities based on DGGE OTUs distributed communities along the gradient of Cd concentration defined by the first axis (54% of the total variance), while the second axis (12% of the total variance) separated control communities from all the other treatments, and further separated communities exposed to mixtures of phenanthrene with increasing Cd concentrations (Fig. 3B).

Discussion

In the current study, the exposure of freshwater microbial decomposer communities to high Cd concentrations ($\geq$ 3.6 mg L^{-1}) led to a reduction in leaf decomposition. This

might be explained by the reported negative effects of Cd on the activity of fungal extracellular enzymes that degrade plant cell-wall polymers (Baldrian et al. 1996). A poor use of resources were consistent with the observed decrease in fungal activity as sporulation rates in treatments with the highest Cd concentrations, particularly in the presence of phenanthrene. In previous studies concentrations of Cd up to 0.1 mg L^{-1} inhibited both growth and sporulation of aquatic hyphomycetes (Abel and Bärlocher 1984) and 1.5 mg L^{-1} of Cd inhibited leaf decomposition by three strains of aquatic hyphomycetes isolated from a clean site (Fernandes 2008). Although there are no studies in literature about the joint effects of metals and PAHs on aquatic microbially mediated processes, Cd and phenanthrene are reported to have variable effects on organisms depending on the concentration and magnitude of interactions between toxins (Moreau et al. 1999). For instance, the exposure to a mixture of the two toxins resulted in antagonistic effects on the feeding rate of the oligochaete *Ilyodrilus templetoni* (Gust and Fleeger 2006). In another study, Cd combined with phenanthrene decreased the grazing rates of the copepod *Schizopera knabeni* but no interactive effects on feeding were found (Silva et al. 2009). In soils, the addition of heavy metals and PAHs, including Cd and phenanthrene, led to a greater biocidal effect on microbes when toxins were amended together than alone (Maliszewska-Kordybach and Smreczak 2003; Shen et al. 2005). In our study, phenanthrene appeared to accentuate the negative effects of Cd because the highest inhibitions of fungal biomass and sporulation rates were in treatments with both toxins.

The inhibitions of fungal biomass and sporulation after exposure to the toxins were probably due to the fact that aquatic fungi were channeling part of the energy available for growth and reproduction to the synthesis of compounds and/or enzymes involved in cellular detoxification processes. Indeed, aquatic hyphomycetes are reported to be able

to trigger defense mechanisms against heavy-metal exposure by increasing the activity of antioxidant enzymes (Azevedo et al. 2007, 2009; Braha et al. 2007) or the production of thiol-containing compounds that are able to sequester metal ions or scavenge ROS (Guimarães-Soares et al. 2007; Jaeckel et al. 2005; Miersch et al. 1997). Other possible responses of fungi to heavy metals include the secretion of organic acids, polysaccharides, melanin or proteins capable of complexing and / or precipitate metals, regulation of the transport of metalic, adsorption of metals to the cell wall, and its chemical transformation and cellular compartmentalization (Baldrian 2003, Blaudez et al. 2000). On the other hand, there are studies reporting that white rot fungi are able to metabolize PAHs, including phenanthrene, by the phase I enzymes cytochrome P-450 monooxygenase and epoxide hydrolase (Bezalel et al. 1996, 1997; Capotorti et al. 2004). Other enzymes, such as manganese peroxidase, are also involved in the degradation of phenanthrene by white-rot fungi (Baborová et al. 2006). However, the addition of phenanthrene led to a more pronounced effect of Cd on the activity of several enzymes produced by soil microorganisms (Shen et al. 2005). We also noticed that Cd alone (1.2 mg L^{-1}) stimulated biomass production. Other studies revealed that the growth of aquatic hyphomycetes was less sensitive than sporulation to metals (Abel and Bärlocher 1984, Duarte et al. 2004, 2008a, 2009). Probably fungi limit their sporulation and increase their growth in these conditions as a defense mechanism. Moreover, bacterial biomass on decomposing leaves is also reported to be severely inhibited in the presence of metals (Duarte et al. 2008a, 2009). Antagonistic interactions between fungi and bacteria are reported to occur during leaf decomposition in streams (Mille-Lindblom and Tranvik 2003); both groups of microorganisms can compete for resources and thus if bacteria are more sensitive to metals, fungi might take advantage and increase its biomass.

A decrease in aquatic hyphomycete diversity has been found in metal-polluted streams (Bermingham et al. 1996; Niyogi et al. 2002; Pascoal et al. 2005b; Sridhar et al. 2001). Also, in our study, the exposure to increasing concentrations of Cd caused a decrease in the number of fungal taxa, assessed as conidium morphotypes or DGGE OTUs, with the highest declines in treatments with the highest Cd concentrations and when both toxins were added together (ca. 2- to 3-times lower than control microcosms). However, the effects did not appear to be so drastic when diversity was assessed based on DGGE. This is not surprising since analysis based on DNA offers the advantage of detecting species from non-sporulating mycelia and sporulation has been consistently found to be very sensitive to pollutants (Duarte et al. 2004, 2008a; Niyogi et al. 2002; Sridhar et al. 2001, 2005).

The exposure to toxins also induced shifts in the structure of fungal community revealed by multivariate analyses based on both sporulating species or OTUs. In this and other studies, microbial communities exposed to high metal concentrations or mixtures of pollutants were most different from control communities (Duarte et al. 2004, 2008a, 2009). In our study, the dominance pattern of sporulating fungal species was affected by exposure to both toxins; the dominant species in control, *Articulospora tetracladia*, showed high sensitivity to toxics similar to that found in field observations (Duarte et al. 2008b; Pascoal et al. 2005b), while *Alatospora pulchella* was the most tolerant species to Cd, even in the presence of phenanthrene. Some species of aquatic hyphomycetes, such as *Heliscus lugdunensis* and *Flagellospora curta*, are reported to tolerate high levels of Cd (Azevedo and Cássio 2010; Braha et al. 2007; Guimarães-Soares et al. 2007) or to metabolize PAHs such as naphtol (Augustin et al. 2006). Interestingly, in our study, some species were even stimulated by phenanthrene, as it was the case of the unknown species, which had increased spore production with

exposure to phenanthrene. Overall, results show that the presence of phenanthrene accentuated the deleterious effects of Cd on the diversity and activity of aquatic fungal decomposers. Experiments using multiple stressors might correspond to more realistic approaches to assess the effects of pollutants on aquatic biodiversity and stream ecosystem functioning. In the future, more experiments are needed to better understand the interactions between Cd, phenanthrene and other stressors and to predict their impacts on aquatic detritus foodwebs.

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Table 1. Mean percentage contribution of individual fungal species on decomposing leaves to overall conidia produced after 10 and 14 days of exposure to Cd and Phe alone or in mixtures. Treatments, Cd1: 0.06 mg L^{-1}, Cd2: 0.6 mg L^{-1}, Cd3: 1.2 mg L^{-1}, Cd4: 3.6 mg L^{-1}, Cd5: 4.5 mg L^{-1}, Phe: 0.2 mg L^{-1}, Ct: control. Abb, species abbreviation.

Abb	Species	% of conidia											
		Ct	Cd1	Cd2	Cd3	Cd4	Cd5	Phe	Cd1Phe	Cd2Phe	Cd3Phe	Cd4Phe	Cd5Phe
AA	*Alatospora acuminata* Ingold	1.8	1.7	3.6	2.7	10.4	1.0	13.0	-	0.1	0.5	-	-
AP	*Alatospora pulchella* Marvanová	20.4	34.4	63.4	57.7	33.0	31.8	29.8	41.5	72.8	88.6	39.8	54.2
AF	*Anguillospora filiformis* Greath.	0.5	0.1	-	-	-	-	-	-	-	-	-	-
AT	*Articulospora tetracladia* Ingold	40.0	19.8	14.7	1.4	4.1	17.7	7.7	15.1	2.1	0.7	-	6.3
CA	*Clavariopsis aquatica* De Wild.	2.0	0.1	<0.1	0.5	-	0.2	-	-	-	-	-	-
CL	*Clavatospora longibrachiata* (Ingold) Marvanová & Sv. Nilsson	15.9	36.6	2.8	0.1	0.6	-	-	-	-	-	-	-
DF	*Dimorphospora foliicola* Tubaki	-	-	-	-	-	-	0.8	0.1	-	-	-	-
LA	*Lemonniera aquatica* De Wild.	0.9	0.9	1.7	5.8	28.5	41.3	-	1.5	7.1	2.1	22.2	4.2
LC	*Lunulospora curvula* Ingold	0.2	0.3	<0.1	-	-	-	-	-	-	-	-	-
TE	*Tetrachaetum elegans* Ingold	6.6	4.1	12.2	27.5	12.8	-	33.7	38.5	16.3	5.4	2.3	4.2
TB	*Tetracladium breve* A. Roldán	0.1	0.1	0.4	0.1	-	-	0.6	-	-	-	-	-
TSt	*Tetracladium setigerum* (Grove) Ingold	-	-	-	-	-	0.2	0.8	-	-	-	-	-
TSp	*Tricladium splendens* Ingold	1.3	1.0	0.5	0.6	1.6	0.5	2.2	0.8	0.1	1.3	4.0	-
TA	*Triscelophorus cf. acuminatus* Nawawi	0.3	-	-	-	-	-	-	-	-	-	-	-
Un	Unknown branched species (50-30 µm)	0.4	0.2	0.7	-	-	7.5	0.1	2.4	1.1	1.5	31.8	31.3
S1	Sigmoid 1 (80-2 µm)	0.3	0.1	-	0.1	0.2	-	-	-	-	-	-	-
S2	Sigmoid 2 (50-1.5 µm)	0.2	0.1	-	-	-	-	-	-	-	-	-	-
S3	Sigmoid 3 (20-4 µm)	9.4	0.1	-	3.7	9.0	-	11.4	0.2	0.2	-	-	-
S4	Sigmoid 4 (110-1.5 µm)	<0.1	0.2	0.1	-	-	-	-	-	0.1	-	-	-
	Nº of conidial morphotypes	17	16	12	11	9	8	10	8	9	7	5	5
	Nº of DGGE OTUs	18	19	16	14	14	13	13	12	12	10	10	10

Figure legends

Figure 1. Effects of Cd and / or Phe on leaf mass loss (A), fungal biomass (B) and fungal sporulation (C). Leaf mass loss and fungal biomass were measured after 14 days of exposure, while fungal sporulation was measured after 10 and 14 days. n=3, error bars indicate ± 1 SEM; *: $p<0.05$.Treatments, Cd1: 0.06 mg L^{-1}, Cd2: 0.6 mg L^{-1}, Cd3: 1.2 mg L^{-1}, Cd4: 3.6 mg L^{-1}, Cd5: 4.5 mg L^{-1}, Phe: 0.2 mg L^{-1}. Control: 0,061±0,009 g

Figure 2. DGGE fingerprints of the ITS2 region of rDNA of fungal communities on decomposing leaves. M, mixture of DNA from pure cultures. AT, *Articulospora tetracladia*; VE, *Varicosporium elodeae*; AA, *Alatospora acuminata*, LA, *Lemonniera aquatica* and TC, *Tricladium chaetocladium*. Treatments, Cd1: 0.06 mg L^{-1}, Cd2: 0.6 mg L^{-1}, Cd3: 1.2 mg L^{-1}, Cd4: 3.6 mg L^{-1}, Cd5: 4.5 mg L^{-1}, Phe: 0.2 mg L^{-1}.

Figure 3. Correspondence Analysis (CA) diagrams for ordination of fungal communities based on the 19 sporulating species (A) and on the 24 DGGE OTUs (B) exposed to Cd and/or Phe for 14 days in microcosms. See Table 1 for species names and abbreviations in A. Treatments, Cd1: 0.06 mg L^{-1}, Cd2: 0.6 mg L^{-1}, Cd3: 1.2 mg L^{-1}, Cd4: 3.6 mg L^{-1}, Cd5: 4.5 mg L^{-1}, Phe: 0.2 mg L^{-1}